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31EG4 mammary epithelial cells were grown to confluence on filters, and apoptosis was initiated by staurosporine and mitomycin (kinase inhibitors) and also by thapsigargin (releases Ca stores by blocking Ca pump) and collagenase IV (degrades extracellular matrix). As shown by specific staining with fluorescent dyes, apoptosis began to occur in a small percentage of cells (after 16 hrs) as shown by condensation and fragmentation of the nuclei accompanied by extracellular membrane appearance of phosphatidylyserine (annexin V staining). After 48 hrs, a majority of cells were undergoing apoptosis or had been killed. Although cytosolic [Ca] was the same in both control and apoptosing cells, stores of Ca (likely in the ER) were more than 50% smaller in the apoptosing cells compared to controls. As shown by deconvolution microsopy, these apoptosing cells exhibited prominent stores of Ca in the center of the fragmenting nucleus, which appeared to surround the store. These results may indicate an intimate relationship between the store and nuclear fragmentation. Further studies using electrophysiolgical methods showed that control mammary epithelial cells exhibit polarized Na absorption and Cl secretion that could contribute to control of milk secretion and also to fluid accumulation in mammary cysts.

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FOREWORD

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INTRODUCTION

The goal of this research was to determine the potential interactive roles of Bcl-2 and cytosolic and organelle (particularly mitochondria and endoplasmic reticulum) Ca in regulation of apoptosis in mammary epithelial cells. We have been attempting to study these topics in a normal (ie, non-cancerous) mouse mammary cell line using digital imaging methods to measure cytosolic and organelle Ca and immunomicroscopy to identify Bcl-2 expression. Cells were treated with a variety of agents to induce apoptosis, and Ca signaling and cell morphology was studied at various stages. One of our goals was to attempt to utilize cells grown under physiological conditions, i.e., on filters with defined apical and basolateral surfaces. This lead to unexpected difficulties in making Ca measurements, and this slowed progress considerably. However, growing the cells in this physiological state also lead to some interesting, unanticipated electrophysiological data that were obtained in collaboration with another researcher on the Berkeley campus investigating the role of ion channels in secretion of fluids by mammary cysts.

Body

Technical objective #1: Stable transfection of 31EG4 cells with Bcl-2 gene.

This proved to be more difficult than anticipated. We obtained a Bcl-2 plasmid from Gabriel Nunez (U. Michigan) and spent more than one year trying to transfect the cells with no success. We tried calcium phosphate precipitation and This involved using anti-Bcl-2 antibodies and several other types of cells that normally express the protein to confirm that our antibodies and techniques were working properly. In the end we concluded that the Bcl-2 overexpression was preventing the cells from dividing at sufficient rate to generate a stable transfected line. We decided to discontinue this line of research and concentrate solely on obtaining data about the Ca signaling pathways in 31EG4 cells.

Technical objective #2: Activation and determination of time course of apoptosis in control and Bcl-2-transfected cells.

2a. Induction of apoptosis with different reagents. Experiments were performed on 31EG4 cells grown on filters coated with collagen to enhance differentiation of the cells into polarized monolayers with adequate transepithelial resistance. We found that staurosporine and mitomycin C (protein kinase inhibitors), thapsigargin (blocks Ca pump in ER and releases store Ca) and collagenase IV (breaks down matrix used to bind cells to basement membrane) all caused cells to undergo a characteristic pattern of apoptosis, which was monitored using immunomicroscopy and other morphological markers:

Fluorescent annexin V (binds to phosphatidylserine) was used on living cells to identify cells that were beginning apoptosis. Simultaneous labeling with the nuclear stain Syto-11 (yellow) showed that annexin V staining (green or red) began at a time when the nucleus was just beginning to condense and form the characteristic fragmented appearance. This also coincided with a condensation of the nuclear material, leading to brighter staining with Syto-11. When cells finally died, they took up the impermeant nuclear stain propidium iodide (red) and also exhibited staining with annexin V throughout the cell (as opposed to only the outsides of the cells). Results from these experiments showed the time course of apoptosis: After about 16 hrs, 1-5% of the cells were undergoing apoptosis, but there was little cell death. After 48 hrs, most cells had undergone apoptosis and >50% of the cells had died. Because of the use of the vital stains Syto-11 and propidium iodide we were able to determine when cells were apoptosing and then dying so that measurements of cell [Ca] could be performed on single, living control and apoptosing cells simultaneously.

2b. Electrophysiology of mammary epithelial cell ion channels. During the course of these studies we attempted to utilize changes in the transepithelial resistance of the monolayers as an easy and rapid measure of the time course of apoptosis (rather than using the fluorescent stains). We discovered that 31EG4 cell monolayers generated transepithelial ion absorption and secretion processes that were similar to those being measured in the same cells by our colleague Sheldon Miller (MCB Dept, Univ. Calif.-Berkeley), who has been studying ion transport in mammary epithelial cell monolayers and the formation of mammary cysts. We have now completed a fairly comprehensive study of the roles of ion channels and tight junctions in the absorption and secretion of fluids by these mammary cells.

Transepithelial electrophysiology showed amiloride-sensitive cation absorption currents and forskolin- or cAMP-stimulated anion secretion currents; microelectrode experiments showed amiloride-sensitive and forskolin-sensitive changes in apical membrane potential and resistance consistent with the presence of apical ENaC and CFTR; patch clamp measurements showed amiloride-inhibited whole cell Na currents and forskolin-stimulated whole cell Cl currents. Fluid transport measurements showed fluid absorption that was blocked by apical amiloride and fluid secretion that was stimulated by forskolin. Equivalent circuit analysis showed that the cells had relatively leaky junctions. We concluded that 31EG4 cell monolayers absorbed Na (through the cells) and an accompanying anion (through the tight junctions) and fluid (by osmosis, likely through the cells). The Na enters the cells through an apical ENaC channel and likely exit the cell through a Na/K-ATPase, similar to many other epithelia. During stimulation with agents that elevated cellular cAMP, the cells secreted Cl (through the cells, which have apical CFTR), and Na followed the secreted Cl through the tight junctions while fluid was secreted across the cells by osmosis. These results showed that mammary duct cells can help to control the fluid

composition of milk by either secreting or absorbing ions and fluid. These results are also important in showing the ion transport pathways that may be involved in secreting fluids into mammary cysts. This paper is now being completed for submission to the American Journal of Physiology for publication.

We also discovered using patch clamp methods that 31EG4 cells express high levels (judged electrophysiologically) of an outwardly rectifying K channel. This channel had very low conductance at voltages below about –20 mV (inside negative), but increased dramatically at more depolarized cell potentials. This K conductance could potentially play a role in apoptosis, leading to loss of K and accompanying anions and fluid, thereby contributing to the large reduction in cell size observed in our experiments. For example, depolarization of the cells as the cells gradually begin to die would lead to activation of the K channels. Further experiments to investigate the role of this K channel in function of mammary epithelial cells in control and apoptosis will be pursued using other funding.

Technical objective #3: Measurement of cytosolic, ER and mitochondrial [Ca] before and during apoptosis.

31EG4 cells were grown on filters and treated with staurosporine to induce apoptosis. Ca signaling was measured during control conditions and again the next day in cells which were undergoing obvious apoptosis.

The measurements of cytosolic [Ca] in polarized cells grown as monolayers turned out to be more difficult than originally thought. A method was developed for growing cells on the "bottoms" of the filters to allow use of our inverted microscope. A long working, water immersion lens was used, but, due to its relatively poor optical properties (compared to our other Neofluar objectives), a lengthy set of control experiments was performed to assure that differences in fura-2 ratios could be interpreted accurately in terms of differences in cytosolic [Ca]. This chamber is now being used for all subsequent studies of Ca signaling in mammary and other epithelial cells.

Experiments on both control and apoptosing cells showed that cytosolic [Ca] = 50 - 100 nM, and this was quite well regulated, even in the apoptosing cells. Treatment with thapsigargin was used to show the size of the store present in the endoplasmic reticulum, and, though we are still analyzing some of the experiments, presently available data showed that the Ca store was reduced by at least 50% during apoptosis. Interestingly, the apoptosing cells showed local regions ("hot spots") of very high [Ca], likely in the range of concentrations expected to be found in the ER (> 100 μM).

Deconvolution microscopy studies (performed in collaboration with John Flannery, Optometry School, Univ. Calif. Berkeley) showed that these small hot spots of Ca stores (shown with fura-2) were surrounded quite selectively by the degenerating, segmenting nucleus (shown with Syto-11 staining). We are presently analyzing these data further to determine the relationships between the degrading nucleus and the ER store of Ca. This finding may have implications for the interactions between store Ca and the characteristic breakdown of the nucleus in apoptosis. These deconvolution micrographs are presently being analyzed to accompany the Ca signaling data in a paper to be submitted for publication.

Due to the difficulties in accomplishing the above studies, we did not perform experiments designed to test the role of mitochondrial Ca in apoptosis.